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ORIGINAL ARTICLE

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Cotton-fiber germin-like protein. II: Immunolocalization, purification, and functional analysis

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Abstract Cotton (*Gossypium hirsutum* L.) contains a germin-like protein (GLP), *Gh*GLP1, that shows tissue-specific accumulation in fiber. The fiber GLP is an oligomeric, glycosylated protein with a subunit size of approximately 25.5 kDa. Accumulation of *Gh*GLP1 occurs during the period of fiber elongation [4–14 days post-anthesis (DPA)]. During early phases of fiber development (2–4 DPA), *Gh*GLP1 localizes to cytoplasmic vesicles as shown by confocal immunofluorescent microscopy. In slightly older fibers (7–10 DPA), *Gh*GLP1 localizes to the apoplast. In other plants, germins and GLPs have been reported to have enzymatic activities including oxalate oxidase (OxO), superoxide dismutase, and ADP-glucose pyrophosphatase. Cotton fiber extracts did not contain OxO activity, nor did intact fibers stain for OxO activity. A four-step purification protocol involving ammonium sulfate precipitation of a 1.0 M NaCl extract, ion-exchange chromatography on DEAE-Trisacryl M, lectin-affinity chromatography, and gel filtration chromatography resulted in electrophoretically pure *Gh*GLP1. While 1.0 M NaCl extracts from 10–14 DPA fiber contained superoxide dismutase and phosphodiesterase activities, *Gh*GLP1 could be separated from both enzyme activities by the purification protocol. Although a GLP accumulates in the cotton fiber apoplast during cell elongation, the function of this protein in fiber growth and development remains unknown.

Keywords ADP-glucose pyrophosphatase/phosphodiesterase · Cell wall · Germin-like protein · *Gossypium hirsutum* · Oxalate oxidase · Superoxide dismutase

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Abbreviations *ABP* Auxin binding protein · *AGPPase* ADP-Glucose pyrophosphatase/phosphodiesterase · *bis-PNPP* Bis-*p*-nitrophenol phosphate · *ConA* Concanavalin A · *DOA* Day of anthesis · *DPA* Days post-anthesis · *GLP* Germin-like protein · *Mn-SOD* Manganese superoxide dismutase · *OxO* Oxalate oxidase · *PBS* Phosphate-buffered saline

Introduction

Cotton (*Gossypium hirsutum* L.) fibers are single-cell trichomes that differentiate from epidermal cells of developing cotton ovules. Fiber cell elongation occurs for a 3-week period uninterrupted by cell division. Secondary cell wall thickening commences before the cell has completely elongated and results in over 90% of the mature fiber weight being composed of highly crystalline cellulose.

In an effort to clone developmentally regulated genes that are fiber-specific in their expression patterns, a differential display screen (Liang and Pardee 1991) was conducted, comparing wild type cotton, TM1 (Kohel et al. 1970), and a near-isogenic naked seed mutant, N1 (Kim and Triplett 2003). Nucleotide analysis of one of the differential display amplification products showed sequence similarity to members of the cupin gene superfamily (Kim and Triplett 2003). Cupins are characterized by their conserved β-barrel structure but are functionally diverse proteins (Dunwell 1998; Dunwell et al. 2001; Khuri et al. 2001). The first protein to be described in this superfamily was wheat germin (Thompson and Lane 1980; Grzelczak and Lane 1984). Germin protein accumulation is developmentally regulated during germination in wheat and other cereals (Thompson and Lane 1980; Grzelczak et al. 1985). Due to nucleotide sequence similarity with oxalate oxidase

(OxO), wheat germins were tested for enzyme activity and found to be able to convert oxalate to carbon dioxide and hydrogen peroxide (Lane et al. 1993; Lane 2002). Wheat germin is a homohexamer of 22.1 kDa glycosylated subunits (McCubbin et al. 1987; Woo et al. 1998) and is highly resistant to protease activity (Grzelczak and Lane 1984).

In addition to the "true germins" found in monocots, related proteins called germin-like proteins (GLPs) are found in dicots, monocots, and gymnosperms (Hurkman et al. 1991; Heintzen et al. 1994; Domon et al. 1995). Proteins with sequence similarity to germins are not limited to the plant kingdom. The first proteins reported to have substantial sequence identity to germin were spherulins; proteins produced by the slime mold *Physarum polycephalum* during plasmodial encystment (Lane et al. 1991). GLPs share 30–70% sequence identity with "true germins" (Bernier and Berna 2001), notably in the region of two histidine-containing motifs that are involved with Mn^{2+}-binding (Requena and Bornemann 1999). Many proteins of microbial origin also contain these conserved domains (Dunwell et al. 2000; Khuri et al. 2001). The cupin domain is comprised of the two conserved, histidine-containing motifs and a more variable region between the two motifs. Germins and GLPs contain one cupin domain, whereas other members of the cupin superfamily, such as fungal oxalate decarboxylase, soybean sucrose-binding protein, and seed storage globulins, contain two cupin domains (Dunwell 1998).

More than 100 GLPs have been reported in public databases; however, most are known only as putative proteins deduced from nucleotide sequences. Only a few GLPs have been characterized biochemically, and none have OxO activity. Peach auxin-binding proteins (ABPs) were purified by 2,4-D affinity chromatography and found to have sequence similarity to GLPs (Ohmiya et al. 1998). Later, GLPs from a moss (Yamahara et al. 1999) and from tobacco nectary tissue (Carter and Thornburg 2000) were reported to function as superoxide dismutases (SOD). Soon afterward, barley germin was reported to be bifunctional, exhibiting both OxO and SOD activities (Woo et al. 2000). However, several other laboratories have been unable to substantiate the claim of germin bifunctionality (Carter and Thornburg 2000; Bernier and Berna 2001; B.G. Lane, personal communication; S. Bornemann, personal communication). Additional functions proposed for GLPs include nucleotide-sugar pyrophosphatase/phosphodiesterase catalyzing the hydrolysis of ADP-glucose to glucose-1-phosphate and AMP (Rodríguez-López et al. 2001) and serine protease inhibitors (Segarra et al. 2003).

It is evident from large-scale sequencing efforts that GLPs are members of multi-gene families. For example, there are nearly 40 *GLP*-related sequences in the *Arabidopsis* genome (TIGR Arabidopsis annotation database; http://www.tigr.org) and expression appears to be tissue-specific for the *Arabidopsis* isotypes examined (Carter et al. 1998; Membré et al. 2000). Nevertheless, the subcellular localization of germins and GLPs in plant cells has received little attention. Immunogold labeling of *Sinapus alba* leaf GLP showed that the protein is predominantly associated with primary cell walls (Heintzen et al. 1994). A similar localization pattern was detected for peach GLPs (Ohmiya et al. 1998). Barley leaf GLP is loosely associated with the cell wall and becomes insoluble as a result of pathogen infection or abiotic stress (Vallélian-Bindschedler et al. 1998). In germinating wheat embryos, 40% of the germin localizes to the cell wall (Lane et al. 1992); however, the location of the remaining germin is unknown (Bernier and Berna 2001).

Our differential display screen identified a cotton fiber *GLP* (*GhGLP1*) whose expression predominated during the cell wall expansion phase of fiber development (Kim and Triplett 2003). In this report we have purified *Gh*GLP1 from developing cotton fibers and tested whether any of the enzymatic activities previously linked with GLPs are associated with the cotton fiber protein. In addition, using antibodies produced against recombinant forms of the *Gh*GLP1 monomer and against a peptide from the C-terminal end of *Gh*GLP1, we have monitored the localization of *Gh*GLP1 in cotton fiber using confocal immunofluorescent microscopy. These studies were designed to improve our understanding of the role of GLPs in plant growth and development, especially in cell wall biogenesis.

Materials and methods

Plant materials

Cotton (*G. hirsutum L.* TM1) plants were grown under standard field conditions in New Orleans, La., during the summers between 1999 and 2002. Immature seeds with fiber attached were harvested from both lines of plants by 9:00 a.m. and were frozen in liquid nitrogen. Developing carpels were collected at 2-day intervals from the day of anthesis (DOA) through 40 days post anthesis (DPA). Hypocotyls, roots and cotyledons were harvested from 10-day-old seedlings. Leaves (5 cm in width), petals, bracts, fibers and seeds were harvested from plants grown in a greenhouse at 25–32°C. All tissues were frozen in liquid nitrogen, and stored at −80°C.

Antibody production

Anti-rGhGLP1 antibodies

The coding region of *GhGLP1* cDNA minus the signal sequence (AF116537) was ligated in frame between the *Eco*RI and *Sal*I sites into pET-29a(+) (Novagen, Madison, Wis.), a plasmid used for expression of 6× His-tagged proteins. The recombinant plasmid was transformed into BL21(DE3) cells containing pLysS. The protein was induced by the addition of isopropyl-*β*-D-thiogalactopyranoside (IPTG) to 1 mM final concentration. The 6× His-tagged recombinant protein, expressed in bacterial inclusion bodies, was purified on a nickel affinity column (Novagen) in the presence of 6 M urea. Purified recombinant protein from bacteria was separated on a 12% SDS polyacrylamide gel. The protein bands were stained with $CuCl_2$ (Lee et al. 1987), cut from the gel, and used

directly as antigen. Purified protein (1 mg) was used to raise rabbit polyclonal antibodies (Antibodies Incorporated, Davis, Calif.).

Anti-C-terminal peptide antibodies

A polypeptide (AQIKKLKGVLGGTG) from the C-terminus of *Gh*GLP1 (AF116537) was synthesized, conjugated to keyhole limpet hemacyanin using 1-ethyl-3-(3-dimethylaminopropyl) carboiimide hydrochloride (Bauminger and Wilchek 1980), and used to raise rabbit polyclonal antibodies (Sigma-Genosys, Woodlands, Tex.). The C-terminal peptide was conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and used for affinity purification of the C-terminal peptide antibodies. Bound antibodies were eluted with 0.2 M glycine, pH 1.8 and neutralized immediately by the addition of one-tenth volume of 1.0 M Tris-HCl, pH 8.0. Bovine serum albumin (0.1%) and NaN_3 (0.01%) were added to stabilize the affinity-purified antibodies for short-term storage at 4°C.

Immunolocalization

Affinity purified antibodies were used to determine the localization pattern of *Gh*GLP1 in developing cotton fiber using confocal microscopy. Fibers were examined either as whole-mounts or as sections. The use of sections ensured that antibody penetration through the cell wall and into the cytoplasm was complete. In all cases, ovules with attached fibers were fixed in a solution of 4.0% formaldehyde (freshly prepared from paraformaldehyde), 5.0% DMSO, and PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgCl_2$). The fixation period was either 2.5 h at room temperature or 12 h at 4°C. Following fixation, the tissue was rinsed in three changes of PHEM buffer for 10 min each. Material destined for sectioning was then embedded and sectioned in Steedman's wax (Brown and Lemmon 1995). Whole-mount material was treated with 0.5% pectinase in PHEM for 15 min, rinsed in three changes of PHEM for 5 min each, extracted in PHEM, 5% DMSO, 1% Triton-X 100 for 1 h, and finally rinsed in PHEM three times for 15 min each. For immunostaining, all materials were incubated in affinity purified primary antibody in phosphate-buffered saline (PBS) for 1 h, rinsed three times in PBS for 5 min each, incubated in goat anti-rabbit secondary antibody conjugated to fluorescent reporters for 1 h, and mounted in MOWIOL4-88 (Calbiochem, San Diego, Calif.) in Tris-HCl, pH 8.5, 1 mg/ml phenylenediamine dihydrochloride. A Bio-Rad MRC 1024 ES confocal microscope was used to view the stained specimens and to acquire images.

Protein extraction and purification

Denatured total protein was phenol-extracted from selected tissues at different developmental stages according to the method of Barent and Elthon (1992). The extraction buffer was composed of 55% water-saturated phenol in 700 mM sucrose, 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 100 mM potassium chloride, 5 mM ethylenediamine tetraacetic acid. After methanol-ammonium acetate precipitation, proteins were collected by centrifugation at 10,000 *g* and resuspended in 1% SDS. Protein content of extracts was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif.).

Fiber extracellular proteins were eluted by bathing whole seeds with associated fibers in 1.0 M NaCl. The ratio of the weight of cottonseed to the volume of 1.0 M NaCl was approximately 1:15 (w/v). Tissues were vacuum infiltrated by three 10 min exposures to a vacuum line (85 kPa), followed by gentle shaking for 16 h at 4°C. Plant material was recovered by filtration through two layers of cheesecloth followed by centrifugation at 10,000 *g* for 15 min. The supernatant liquid containing extracellular protein was concentrated with a Centriprep-10 unit (Amicon, Beverly, Mass.).

Aqueous-soluble proteins were prepared from fiber and stripped ovules by freezing the tissues (500 mg) and thoroughly grinding in a mortar with liquid nitrogen. The powdered tissues were transferred to a disposable plastic vessel containing 2 ml 0.05 M Tris-HCl, pH 7.0. The mixture was gently shaken on a LabQuake shaker at 4°C for 45 min and centrifuged at 10,000 *g* for 5 min. The supernatant liquid was collected and used for OxO activity assays.

For large-scale purification of *Gh*GLP1, extracellular proteins were eluted by vacuum infiltrating whole seeds in 1.0 M NaCl and concentrated by ammonium sulfate precipitation to 90% saturation. The precipitate was dissolved in 20 mM Tris-HCl, pH 8.0, dialyzed against the same buffer, applied to a DEAE-Trisacryl M column (IBF Biotechnics, France) and eluted with a salt gradient (0.05–2 M NaCl). The fractions containing *Gh*GLP1, identified by immunoblot analysis, were applied to a column of concanavalin A (ConA)-agarose (Sigma, St Louis, Mo.), and the adsorbed proteins were eluted with a gradient (0–200 mM) of methyl α-D-mannopyranoside. Fractions containing *Gh*GLP1 were concentrated with a Centricon-50 filtration unit (Amicon).

Immunoblotting

The extracted proteins were separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane in 25 mM Tris base/190 mM glycine/20% methanol at constant voltage (30 V) overnight at room temperature. The filters were blocked in 5% milk powder (Difco) in PBS-T (0.05% Tween 20 in PBS) buffer for 2 h at room temperature, reacted with either the rabbit anti-r*Gh*GLP1 antibody (1:1,000 dilution) or rabbit anti-C terminal peptide antibody (1:1,000 dilution) in blocking buffer overnight at 4°C, washed three times with PBS-T, reacted with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2,000 dilution; Pierce, Rockford, Ill.). The cross-reacting proteins were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions.

Glycoprotein detection

Glycoprotein analysis was carried out with Pro-Q Emerald 300 glycoprotein gel stain kit (Molecular Probes, Eugene, Ore.) according to the manufacturer's instructions. Stained glycoproteins were visualized using a 300 nm UV transilluminator.

Enzyme activity assays

Oxalate oxidase

Total soluble proteins were extracted from developing fibers at two different developmental stages (7 and 18 DPA). Soluble proteins were spotted onto nitrocellulose membranes and two different OxO assays were conducted between pH 3.0 and pH 9.0 (Lane et al. 1993; Dumas et al. 1995). Extracellular proteins extracted by 1.0 M NaCl from elongating fibers (8–12 DPA) were also tested in a spectrophotometric assay for OxO according to the method of Sugiura et al. (1979). All assays were conducted with a negative control where oxalate was left out of the enzyme assay and a positive control, using a commercial source of barley OxO (Sigma; P-6782).

Superoxide dismutase

A qualitative in-gel SOD assay was performed as described by Beauchamp and Fridovich (1971). The extracellular proteins from developing fibers were separated by a 7.5% native polyacrylamide

gel, pH 8.0. The gel was run until a bromophenol marker reached the bottom of the gel. To detect SOD activity, the gel was incubated in 2.45 mM nitroblue tetrazolium for 20 min, followed by immersion for 15 min in a solution containing 28 mM riboflavin in 36 mM Tris-HCl, pH 7.8. The SOD activity in column fractions was measured using cytochrome *c* as the detector and xanthine plus xanthine oxidase as the superoxide generator (Beauchamp and Fridovich 1971)

Phosphodiesterase

The extracellular proteins from developing fibers were separated by a 7.5% native polyacrylamide gel, pH 8.0 run for 1.5 h after the bromophenol blue marker reached the gel bottom. The gel was incubated with 5 mM bis-*p*-nitrophenol phosphate (bis-PNPP) (Sigma) and 5 mM $MgCl_2$ until the *p*-nitrophenol product appeared as a yellow band (Rodríguez-López et al. 2001). The spectrophotometric assay was carried out according to the method of Nishimura and Beevers (1978). Extracellular proteins were also incubated with 10 mM Tris (pH 8.0) containing 1 mM bis-PNPP and 5 mM $MgCl_2$ at 37°C for 60 min, then absorbance at 405 nm was measured. All assays were conducted with a negative control (no protein) and a positive control, phosphodiesterase I (Sigma; P-4506).

Results and discussion

*Gh*GLP1 is a fiber-specific protein

A differential display screen comparing an early stage of cotton fiber development between TM1 (wild type) and N1 (a near isogenic mutant with no fuzz fibers and sparse lint) led to the identification of *GhGLP1* (AF116537). The gene for *GhGLP1* is specifically expressed in cotton fiber and is developmentally regulated (Kim and Triplett 2003). To investigate the pattern of *Gh*GLP1 protein accumulation during fiber development, two antibodies were produced. The first antibody was generated in rabbits against the denatured, recombinant form of *Gh*GLP1 (anti-r*Gh*GLP1). When anti-r*Gh*GLP1 antibodies were used for immunoblot analysis of total proteins extracted from fiber, the pattern of proteins recognized by the antibody was complex. Furthermore, the antibody to r*Gh*GLP1 recognized a protein in developing cotton testae, precluding the use of this antibody preparation for immunoblotting samples where the seed and fiber could not be separated. Since GLPs appear to be multi-gene families in the higher plants that have been examined thus far (Carter et al. 1998; Membré et al. 2000; Druka et al. 2002), and certain portions of GLPs are conserved in other cupins, it is likely that the fiber *Gh*GLP1 protein is one of several members of the cupin superfamily present in *Gossypium*. Other investigators have produced isoform-specific antibodies to the variable region adjacent to the A box motif of GLPs (Membré et al. 2000). However, antibodies produced against peptides corresponding to the variable region might have difficulty recognizing native forms of *Gh*GLP1 since this motif is likely to be deeply buried in the hexameric structure. Models of cupin structure indicate that the C-terminal region is exposed to the outer surface of the homohexamer (Woo et al. 2000). Therefore, we employed an alternative strategy by producing a peptide antibody against the 14 amino acids of the C-terminus (AQIKKLKGVLGGTG) of *Gh*GLP1. We refer to this antibody preparation as anti-C-terminal peptide.

Immunoblotting with anti-C-terminal peptide shows that accumulation of *Gh*GLP1 protein is fiber specific (Fig. 1). This pattern of protein accumulation is consistent with the expression pattern of the *GhGLP1* transcripts (Kim and Triplett 2003). An abundant protein of 25.5 kDa was evident in 12 DPA developing cotton fibers (Fig. 1, lane 1). The very faint band in the 12 DPA stripped seeds (lint fibers removed) probably arises from incomplete removal of fiber, especially a class of very short fibers called fuzz that are distinct from lint fibers (Fig. 1, lane 2). Very faint bands slightly smaller in molecular mass than *Gh*GLP1 were detected in cotyledon, leaf, and bract tissues, (Fig. 1, lanes 5–7), suggesting that GLPs in cotton may exhibit the dual glycosylation patterns that have been demonstrated for wheat germins (Lane et al. 1987). There was no detectable antigen present in cotton roots, hypocotyls, petal tissue, (Fig. 1, lanes 3, 4, 8) or cotton seed testae (data not shown).

*Gh*GLP1 is an extracellular, oligomeric protein

When 0.1% SDS-soluble, 12 DPA cotton-fiber extracellular proteins were separated by SDS-PAGE, migration of *Gh*GLP1 depended on the addition of a reducing agent and on whether the sample had been heated (Fig. 2a). Fully denatured *Gh*GLP1 migrated with an apparent molecular mass of 25.5 kDa on 10% SDS-PAGE (Fig. 2a, lane 1). Under conditions where the reducing agent was omitted and the sample was not heated prior to electrophoresis, protein bands of two different sizes were detected by the anti-C terminal peptide serum. The molecular mass of the major band is estimated to be 108 kDa and the minor band is estimated at 52 kDa (Fig. 2a, lane 2). Anomalous migration on SDS-polyacrylamide gels by germins and GLPs has been reported previously (Lane 2002). Wheat germin is apoplast-associated and is remarkably resistant to

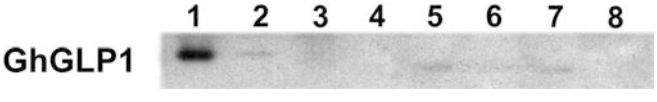

Fig. 1 Fiber-specific accumulation of germin-like protein (GLP) *Gh*GLP1. Total proteins from different tissues were extracted with phenol according to the method of Barent and Elthon (1992); for each lane 4 μg total protein was boiled in SDS sample buffer containing 100 mM DTT and 2% SDS, separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with anti-C-terminal peptide. Lanes: *1* Fibers 12 days post anthesis (DPA), *2* 12 DPA stripped seeds, *3* 10-day-old cotton root, *4* 10-day-old hypocotyls, *5* 10-day-old cotyledons,*6* mature leaf, *7* day of anthesis (DOA) bract, *8* DOA petal

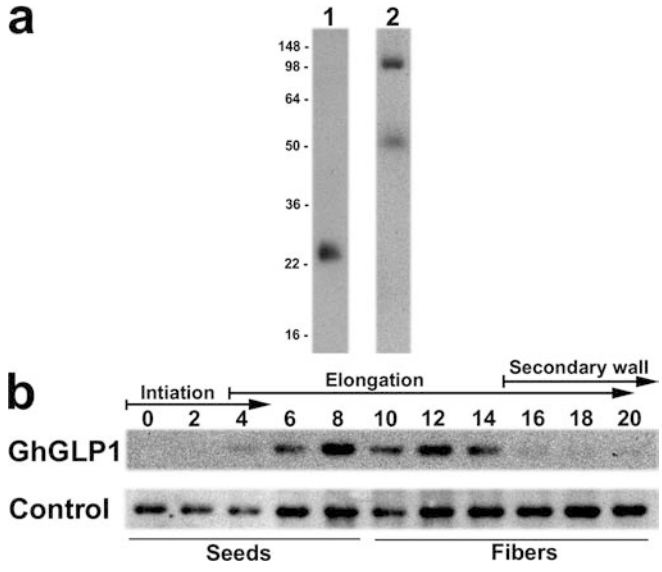

Fig. 2 Immunoblot analysis of *Gh*GLP1 from developing fibers. **a** 10% SDS-PAGE. Lanes: *1* Total fiber proteins (4 µg; 12 DPA fiber) denatured by boiling in the presence SDS sample buffer containing 2% SDS and 100 mM DTT, *2* 2 µg 0.1% SDS-soluble extracellular proteins without reducing agent. **b** Total proteins were extracted from either cottonseeds with their associated fibers (*Seeds*, 0–8 DPA) or fibers separated from seeds (*Fibers*, 10–20 DPA). Immunoblots were carried out as described in Fig. 1

protease degradation and dissociation by detergents (Lane 1991). Originally, germin was hypothesized to be a tetramer or pentamer based on SDS-polyacrylamide gel electrophoresis. It was not until high resolution X-ray diffraction work could be conducted that it was shown that germin is a homohexamer composed of a trimer of dimers (E.F. Pai and B.G. Lane, unpublished, as cited in Gane et al. 1998). In the absence of reducing agent and heat denaturation, fungal-induced barley GLP migrated as two bands on a 12% SDS-polyacrylamide gel with apparent molecular masses of 95 kDa and 100 kDa, even though the predicted size of the deduced homo-hexamer was expected to be 135 kDa (Zhang et al. 1995). After heating and in the presence of dithiothreitol, barley leaf GLP migrated as a prominent band at approximately 50 kDa and fainter bands at 25 kDa and 27 kDa. It is likely that the 50–52 kDa band corresponds to GLP dimers, a form of intermediate stability under denaturing conditions in both cotton fiber and barley leaf samples. Based on similarity with the wheat and barley germins where SDS-PAGE underestimates the molecular mass of the native protein (Lane 2002), the 108 kDa *Gh*GLP1 is also probably a hexamer.

Recovery of *Gh*GLP1 from primary and secondary cell walls

To monitor the accumulation of *Gh*GLP1 protein during fiber development, immunoblot analysis was conducted using total proteins extracted from intact developing seeds with their associated short fibers (0–8 DPA) and fiber cells only (10–20 DPA). Total proteins were extracted from these tissues with a mixture of 50% saturated aqueous phenol and 50% buffer (0.7 M sucrose, 50 mM Tris, 30 mM HCl, 2 mM dithiothreitol, 100 mM KCl, 5 mM EDTA). The phenol-soluble proteins were precipitated with 0.1 M ammonium acetate/methanol (Barent and Elthon 1992). Similar extraction conditions were used previously to extract wheat germin from cell walls (Lane et al. 1992). Since *Gh*GLP1 transcripts were abundant until 14 DPA (Kim and Triplett 2003) and many, though not all, GLPs are unusually resistant to proteolysis, we expected that the level of *Gh*GLP1 protein would remain relatively constant during secondary wall synthesis (>16 DPA). Unexpectedly, the level of extracted *Gh*GLP1 protein increased throughout the fiber cell elongation stage (4–14 DPA) and dropped dramatically with the onset of secondary cell wall synthesis (16 DPA) (Fig. 2b). The apparent reduction in *Gh*GLP1 protein content with the onset of secondary cell wall synthesis could arise either from *Gh*GLP1 becoming insoluble or from selective degradation of *Gh*GLP1 concomitant with secondary wall synthesis. Additional investigations are ongoing to determine if *Gh*GLP1 is degraded or becomes tightly or covalently associated with the cell wall during this time period.

Subcellular localization of *Gh*GLP1

Only a few studies have documented the localization pattern of germins and GLPs. For the true germins, detection of protein localization pattern is possible by cytochemical staining for OxO activity (Dumas et al. 1995; Zhou et al. 1998); however, the resolution of this method is insufficient to determine the precise subcellular location of germin. Immuno-detection methods using alkaline phosphatase-conjugated (Lane 2000) or colloidal gold-conjugated secondary antibody (Lane et al. 1992) show that germin is localized in the cell wall of germinating wheat embryos. Interestingly, tobacco plants expressing wheat germin localize the transgenic gene product in the cell wall (Membré et al. 2000) even though cell wall structure in tobacco is quite distinct from that in wheat. Several approaches have been used to localize GLPs. First, cell fractionation studies suggest that barley leaf GLP (Vallélian-Bindschedler et al. 1998) and some *Arabidopsis* GLPs (Membré et al. 2000) exist in both easily soluble and wall-bound forms. In the two dicots that have been examined by immuno-gold electron microscopy, peach GLP (Ohmiya et al. 1998) and white mustard GLP (Heintzen et al. 1994) localized predominantly in leaf primary cell walls.

Immunofluorescent labeling of 2–4 DPA fiber with anti-C-terminal peptide showed punctate labeling in or near the fiber cell wall and a reduced level of similar labeling in the cytoplasm (Fig. 3a). At this stage of development the cytoplasm has just begun to vacuolate (Fig. 3b). At slightly older developmental stages

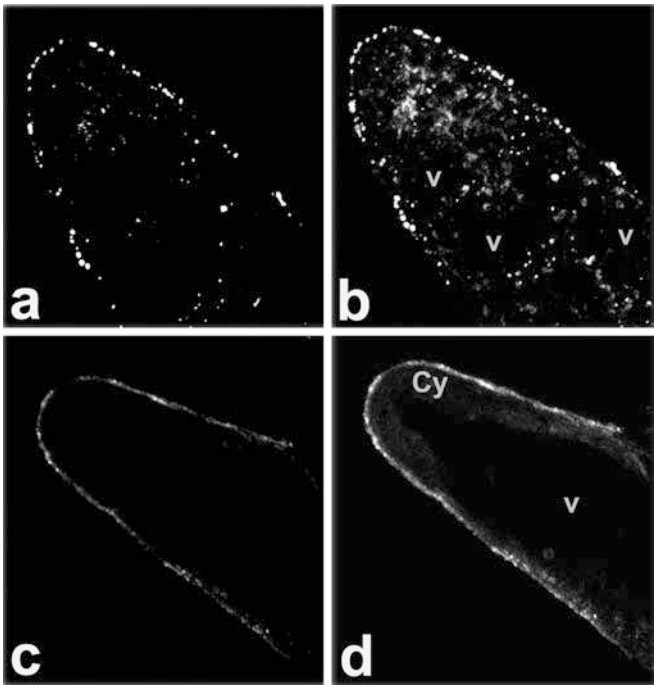

Fig. 3 Immunolocalization of *Gh*GLP1 using anti C-terminal peptide in 2–4 DPA fiber (**a**, **b**) and 7–10 DPA fiber (**c**, **d**). The brightness of plates **b** and **d** was increased to show the degree of vacuole development (*v*) and location of the cytoplasm (*cy*)

(7–9 DPA) fibers show a nearly continuous labeling of the cell wall and little or no staining in the cytoplasm (Fig. 3c). The central vacuole is well developed by this developmental stage (Fig. 3d). When young fibers (2–4 DPA) were double labeled with anti-r*Gh*GLP1 and JIM 84, an antibody that recognizes Golgi bodies (Horsley et al. 1993), there was no overlap in the signals (Fig. 4). This result suggests that cotton GLP localizes to vesicles in the endoplasmic reticulum (ER) during very early stages of fiber development. Since we have not yet conducted a structural analysis of the carbohydrate portion of *Gh*GLP1, it is premature to discuss how the protein may become localized to the apoplast. Another member of the cupin superfamily, maize ABP1, is localized primarily in the ER (Henderson et al. 1997); however, there is disagreement whether ABP1 also co-localizes to Golgi (Jones and Herman, 1993; Henderson et al. 1997).

Oligomeric *Gh*GLP1 is a glycosylated protein

*Gh*GLP1 codes for a 206 amino acid polypeptide with a predicted molecular mass of 21 kDa (with signal peptide) or 19.7 kDa for the mature protein (Kim and Triplett 2003). The molecular mass of *Gh*GLP1 extracted from 12 DPA fibers was estimated to be 25.5 kDa under denaturing conditions (Fig. 2d, lane 1). The molecular mass predicted from the deduced protein is different from the mass estimated by SDS-PAGE and is likely due to glycosylation at a conserved site since all

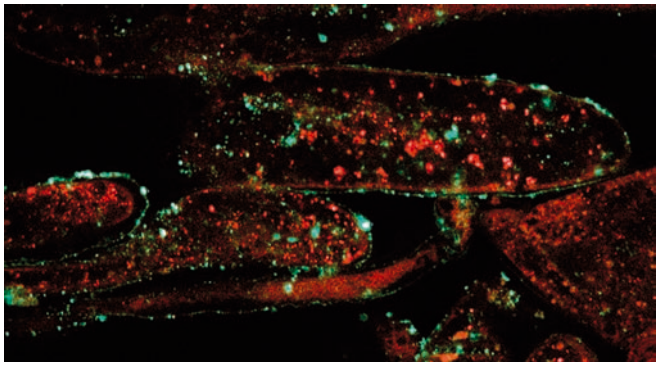

Fig. 4 Simultaneous immunolocalization of *Gh*GLP1 and Golgi bodies in 2–4 DPA fiber using anti r*Gh*GLP1 (*green*) and JIM 84 (*red*) antibodies

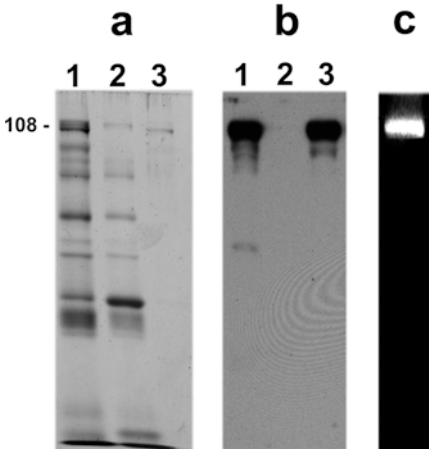

Fig. 5a–c Purification of *Gh*GLP1 from developing cotton fibers. Proteins were electrophoresed on a 10% SDS polyacrylamide gel without heat denaturation or addition of a reducing agent. **a** Silver-stained 10% SDS polyacrylamide gel. Lanes: *1* Peak fractions containing *Gh*GLP1 from DEAE Trisacryl M column, *2* peak fractions not retained by concanavalin A (ConA) (flow through) after loading DEAE-Trisacryl M-purified protein on a Con-A agarose column, *3* peak fractions bound by ConA. **b** Immunoblot analysis. Immunoblotting of a duplicate gel was carried out as described in Fig. 1. **c** Glycosylation of *Gh*GLP1. Peak fractions bound by ConA stained with Pro-QTM Emerald 300 glycoprotein gel stain. Stained glycoprotein was visualized using a 300 nm UV transilluminator

GLPs that have been assayed are glycosylated (Yamahara et al. 1999; Carter and Thornburg, 2000; Membré et al. 2000). Two lines of evidence support this supposition for *Gh*GLP1. First, chromatography of 1 M NaCl-soluble fiber extracellular proteins on DEAE-Trisacryl M (Fig. 5a, b; lane 1) followed by affinity chromatography on Con-A agarose resulted in a preparation yielding a single sharp band at 108 kDa on a silver-stained SDS-polyacrylamide gel when the protein was not heat-denatured or treated with reducing agent (Fig. 5a, b; lane 3). The fiber extracellular proteins that were not retained by ConA-agarose (Fig. 5a, b; lane 2) did not react with the anti-peptide antibody. This highly effective purification of *Gh*GLP1 by ConA-affinity

chromatography is one line of evidence that *Gh*GLP1 has a carbohydrate side-chain containing mannose. Additional evidence for glycosylation comes from staining the ConA-purified protein with Pro-Q Emerald 300 glycoprotein gel stain. A single fluorescent band at 108 kDa was evident (Fig. 5c), indicating unequivocally that oligomeric *Gh*GLP1 is a glycoprotein. Quantitative structural analysis of the carbohydrate portion of *Gh*GLP1 should help clarify whether this protein passes through the Golgi secretory pathway.

*Gh*GLP1 is not an OxO

A variety of enzymatic functions for germins and GLPs have been suggested in the literature, including OxO (Lane et al. 1993, Woo et al. 2000), SOD (Yamahara et al. 1999; Carter and Thornburg 2000, Woo et al. 2000) and ADP-glucose pyrophosphatase/phosphodiesterase (AGPPase) (Rodríguez-López et al. 2001). Although there is high degree of sequence similarity between the true germins and dicot GLPs, only one dicot GLP tested has shown OxO activity (Bringezu et al. 1999).

OxO activity was tested in extracts containing aqueous-extracted total cellular proteins and 1.0 M NaCl-extracted extracellular protein from developing ovules and fibers. Aqueous-soluble proteins from the cell elongation stage (7 DPA) and the secondary cell wall synthesis stage (18 DPA) as well as a positive control, barley OxO from a commercial source, were used. The OxO staining assays were conducted between pH 3.0 and 9.0 (Lane et al. 1993; Dumas et al. 1995). Fiber extracts failed to yield a positive reaction even with extended incubation times and increased amounts of cotton fiber protein (data not shown). Barley OxO gave a positive reaction within a few minutes with both staining procedures. Omission of oxalic acid to the reaction resulted in no color development for the positive control samples. Extracts prepared from stripped ovules also failed to yield a positive reaction in the OxO assay. Cottonseeds at 7 DPA and 18 DPA were placed in the OxO reaction mixtures to stain for activity in situ (Lane et al. 1993; Dumas et al. 1995). Tissue samples did not develop color even after incubating in the staining solution for 1 h. A spectrophotometric OxO assay (Sugiura et al. 1979) with 1.0 M NaCl-soluble cotton fiber extracellular protein showed no OxO activity. These results suggest that cotton fiber *Gh*GLP1, like other GLPs (Vallélian-Bindschedler et al. 1998; Yamahara et al. 1999; Carter and Thornburg 2000; Membré et al. 2000), does not possess OxO activity despite its considerable sequence similarity with wheat and barley germins.

*Gh*GLP1 is not a SOD

Since barley root germin and two GLPs were reported to have Mn-SOD activity (Yamahara et al. 1999; Carter and Thornburg, 2000; Woo et al. 2000), we tested protein preparations from developing cotton fibers for SOD activity. Both in-gel and quantitative SOD assays (Beauchamp and Fridovich 1971) showed that extracellular proteins extracted from cotton fiber contained SOD activity. However, when *Gh*GLP1 was purified by ConA-agarose chromatography, the bound fractions containing the immunoreactive 108-kDa protein did not possess SOD activity. All of the extracellular SOD activity passed through the ConA-column without binding (data not shown). To extend this observation, active forms of cotton-fiber extracellular proteins were subjected to native polyacrylamide gel electrophoresis. Fiber extracellular proteins (1.0 M NaCl-soluble) showed two major active forms of SODs with in-gel activity assays (Fig. 6a, lane 1). An immunoblot of the native gel shows that *Gh*GLP1 has a reduced mobility compared with the two active forms of SOD (Fig. 6a, lane 2). Furthermore, the anti-r*Gh*GLP1 antibody did not detect either of the active SOD forms in an immunoblot of the native PAGE gel, and there is no SOD activity in the region of *Gh*GLP1 in the in-gel assay. As a result, we conclude that extracellular SOD activities are indeed found in developing cotton fibers; however, *Gh*GLP1 can be separated by native polyacrylamide gel electrophoresis and lectin-affinity chromatography from the extracellular SOD activities. This result is consistent with an earlier report in which Mn-SOD was reported to be positioned close to one of the barley root germins on denaturing, two-dimensional gels, but could be distinguished by N-terminal sequencing (Hurkman et al.

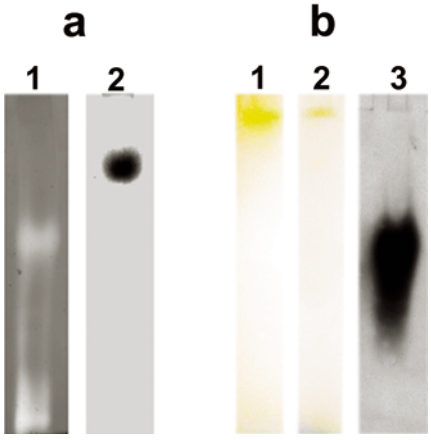

Fig. 6a, b In-gel enzyme activity assays. **a** Superoxide dismutase (SOD) assay. Cotton fiber extracellular protein (10 μg) extracted with 1 M NaCl and concentrated by ammonium sulfate precipitation (90% saturation) was separated on 7.5% native gel run for 1 h. Lanes: *1* In-gel SOD activity assay (Beauchamp and Fridovich 1971), *2* immunoblot analysis. **b** Phosphodiesterase assay. Proteins were separated on a 7.5% native gel run for 2.5 h. Enzyme activity was detected by incubating the gel in 5 mM bis-*p*-nitrophenol phosphate (bis-PNPP) and 5 mM $MgCl_2$ (Rodríguez-López et al. 2001). Lanes: *1* Snake venom phosphodiesterase I (10 mU), *2* 100 μg cotton fiber extracellular proteins, *3* immunoblot of 100 μg cotton fiber extracellular proteins (as *lane 2*) carried out as described in Fig. 1

1991). In this earlier report, the pIs of barley MnSOD and one of the barley germins were identical, with molecular masses of the two proteins differing by 1 kDa. Furthermore, several laboratories have failed to find SOD activity associated with their preparations of true germins or GLPs (Carter and Thornburg 2000; Bernier and Berna, 2001; B.G. Lane personal communication; S. Bornemann, personal communication). Commercial preparations of barley OxO (Sigma P-6782) do not have SOD activity (data not shown). When barley germin was expressed in yeast, a soluble, hexameric recombinant barley germin showed OxO activity, but not SOD activity (Whittaker and Whittaker 2002).

*Gh*GLP1 is not an AGPPase

Recently, barley GLP (*Hv*GLP1) was reported to have AGPPase activity, catalyzing the hydrolytic breakdown of ADP-glucose to produce glucose-1-phosphate and AMP (Rodríguez-López et al. 2001). The primary protein sequence of *Gh*GLP1 is 76% similar to the deduced sequence of *Hv*GLP1 (T05721); therefore, we tested if AGPPase activity exists in cotton fiber extracellular protein preparations. The same phosphodiesterase assay that was used for AGPPase activity by Rodríguez-López and co-workers was used for testing cotton fiber protein preparations (Nishimura and Beevers 1978). Phosphodiesterase activity was found in the fraction of partially purified *Gh*GLP1 after DEAE-Trisacryl M and Con-A agarose columns (data not shown). However, *Gh*GLP1 and the fractions containing phosphodiesterase activities could be separated by gel filtration chromatography (data not shown). It appears that physical characteristics such as pI and glycosylation of cotton-fiber extracellular AGPPase are similar to those of *Gh*GLP1, but the molecular mass of AGPPase is much larger than that of *Gh*GLP1. To further show that *Gh*GLP1 is different from AGPPase, we conducted an in-gel phosphodiesterase assay with cotton fiber extracellular proteins. Unlike barley AGPPases (Rodríguez-López et al. 2001), the phosphodiesterase activity of AGPPase in cotton fibers was not resistant to SDS, so a native gel was used for the in-gel AGPPase assay. Native polyacrylamide gels were incubated in 5 mM bis-PNPP under alkaline conditions to localize proteins on the gel containing AGPPase activity. Yellow bands corresponding to the localized accumulation of *p*-nitrophenol developed in lanes where venom phosphodiesterase I (positive control) and cotton extracellular proteins were loaded (Fig. 6b; lanes 1, 2). Anti-r*Gh*GLP1 antibody detected *Gh*GLP1 about mid-lane in the gel (Fig. 6b, lane 3). Under the electrophoresis conditions used, *Gh*GLP1 showed a much higher mobility than the activity-stained band for extracellular AGPPase (Fig. 6b, lane 3). There was no activity staining in the area where *Gh*GLP1 migrated (Fig. 6b, lane 2). A positive control, venom phosphodiesterase I (Fig. 6b, lane 1) showed the same mobility as cotton fiber extracellular AGPPase. As a result, we conclude that extracellular phosphodiesterase activities exist in developing cotton fibers, but *Gh*GLP1 is not a phosphodiesterase.

Potential functions of *Gh*GLP1 during fiber development

In this paper we document that a GLP, *Gh*GLP1, is distinct from the three enzymatic activities that have been reported previously for cereal germins and GLPs. In addition to the enzymes tested, several other putative functions have been proposed for GLPs. Among the ideas suggested are that GLPs may serve as proteins to facilitate transport of cell-wall polysaccharides (Lane 1991, 1994, 2002), plant cell wall receptors (Swart et al. 1994; Carter and Thornburg 1999; Wisniewski et al. 1999; Membré et al. 2000), ABPs (Ohmiya et al. 1993, 1998), or structural wall proteins (Schweizer et al. 1999). When rhicadhesin, a putative plant receptor molecule for *Agrobacterium* and *Rhizobium* cell surface proteins, was purified from pea root cell walls, the first 29 N-terminal amino acids were 96% identical to *Pisum sativum* GLP (AJ250832) (Swart et al. 1994). The presence of an RGD tripeptide in rhicadhesin suggested that protein-protein interaction between the pea root GLP and the bacterial cell surface proteins could be mediated in a way similar to the function of the RGD motif in animal adhesion proteins like fibronectin and vitronectin (Swart et al. 1994). More than half of the GLPs, but not the ''true'' germins, contain an RGD-like tripeptide (RGD, KGD, or KGE) at the C-terminus (Carter and Thornburg, 1999; Bernier and Berna 2001). The deduced primary sequence of *Gh*GLP1 does not contain the RGD motif, but instead codes for the tripeptide EGD (Kim and Triplett 2003).

Evidence supporting the notion that GLPs may function as structural cell wall proteins comes from analysis of barley GLP following biotic or abiotic stresses (Vallélian-Bindscheller et al. 1998). Heat, H_2O_2 treatment, or pathogen infection led to barley GLP becoming more difficult to extract from leaf cell walls. Prior to stress application, GLP was easily soluble in citrate-phosphate buffer, pH 2.8. After stress application, GLP was removed only by boiling in 1% SDS, suggesting a hydrophobic interaction between barley GLP and some other wall polymer or protein. We are investigating whether insolubility of *Gh*GLP1 may occur developmentally in cotton fiber to explain why the recovery of this protein from fibers older than 16 DPA was low. Nevertheless, insolubility of GLPs does not appear to be a universal phenomenon since white lupin suspension-cultured cells released GLP to the culture medium upon exposure to $CuCl_2$ or fungal elicitors (Wojtaszek et al. 1997). Conversely, it may be argued that suspension-cultured cells are incapable of insolubilizing wall proteins since the apoplast is constantly

bathed in medium. If GLPs function as wall structural proteins, then they represent a distinct class of wall-associated protein since they are not composed of the repetitive elements characteristic of the hydroxyproline, proline, and glycine-rich cell wall proteins (Casseb 1998).

Of the >100 GLP sequences in GenBank, the nucleotide sequence of *Gh*GLP1 is most similar to one of the peach GLPs (U81162) originally isolated as an ABP by 2,4-D affinity chromatography. The peach GLPs (Ohmiya et al. 1993, 1998) have a much lower affinity for naturally-occurring auxins than the maize ABP1. Recently, X-ray crystallography of maize ABP1 dimers revealed striking structural similarity with barley germin dimers despite having only 24% sequence identity (Woo et al. 2002). Therefore, it was suggested that some GLPs might function as regulatory proteins involved in auxin metabolism (Khuri et al. 2001). Interestingly, maize ABP1 is detected at the endoplasmic reticulum, the plasma membrane and the cell wall by immuno-gold labeling (Jones and Herman 1993). The importance of auxin for cotton fiber development has been recognized for many years (Beasley and Ting 1973). The IAA content of young, elongating fibers is high and progressively decreases as the fiber begins accumulating cellulose in the secondary cell wall (Yang et al. 2001). Expression of *Gh*GLP1 was at its highest level during the peak period of fiber expansion, followed by a sharp decline when the rate of expansion slows considerably (Kim and Triplett 2003). The same expression patterns have been found for other genes involved in cotton fiber expansion and elongation (John and Crow 1992; Rinehart et al. 1996; Song and Allen 1997; Smart et al. 1998; Whittaker and Triplett 1999). Based on the sequence similarity to peach GLP/ABP and the expression patterns of *Gh*GLP1, it is possible that *Gh*GLP1 may be involved in auxin-regulated fiber elongation and cell wall expansion. The precise biochemical role of GLPs as putative mediators in this process, however, remains to be determined.

Whether *Gh*GLP1 functions as an extracellular ABP with low affinity for auxin, as a wall structural protein, as a cell wall receptor, or some other, as yet undetermined, function, gene expression and protein accumulation profiles suggest that *Gh*GLP1 could be a participant in developmentally regulated plant cell wall expansion. Continuing efforts to deduce the function of this fiber-specific protein should contribute to our fundamental understanding of the biochemistry of plant cell enlargement.

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